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## **Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer**

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**Title:** Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer

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**At a Glance Commentary:****Scientific Knowledge on the Subject**

Mesenchymal stromal cells (MSCs) are promising candidates for cell based therapy for the Acute Respiratory Distress Syndrome (ARDS). MSC-derived extracellular vesicles (EVs) have been shown to recapitulate many of the beneficial effects of MSCs in lung injury *in vivo*. These MSC-EVs have also been shown to transfer mitochondria to other cell types including macrophages, improving their bioenergetics. The effect of this EV-mediated mitochondrial transfer on macrophage function in the context of ARDS is currently unknown.

**What This Study Adds to the Field**

Human MSCs suppress pro-inflammatory cytokine secretion, enhance phagocytic capacity and promote M2 macrophage marker expression in human macrophages in the presence of bronchoalveolar lavage fluid from ARDS patients through paracrine mechanisms. These effects were mediated by mitochondrial transfer from MSCs to macrophages via EVs and were critically dependent on the enhancement of oxidative phosphorylation in macrophages. Moreover, adoptive transfer of murine alveolar macrophages (AMs) which had been pre-treated with MSC-EVs *ex vivo* mitigated endotoxin-induced lung injury *in vivo*. This study demonstrates an important role of MSC-EVs for MSC effects in ARDS, reveals a new mechanism of macrophage polarization and highlights the essential role of AMs as mediators of MSC therapeutic effects.

This article has an online data supplement, which is accessible from this issue's table of content online at [www.atsjournals.org](http://www.atsjournals.org)

## Abstract

**Rationale:** Acute Respiratory Distress Syndrome (ARDS) remains a major cause of respiratory failure in critically ill patients. Mesenchymal Stromal Cells (MSCs) are a promising candidate for a cell based therapy. However, the mechanisms of MSCs effects in ARDS are not well understood. Here we focused on the paracrine effect of MSCs on macrophage polarization and the role of extracellular vesicle (EV)–mediated mitochondrial transfer.

**Objectives:** To determine the effects of human MSCs on macrophage function in the ARDS environment and to elucidate the mechanisms of these effects.

**Methods:** Human monocyte-derived macrophages (MDMs) were studied in non-contact co-culture with human MSCs when stimulated with lipopolysaccharide (LPS) or bronchoalveolar lavage fluid (BALF) from ARDS patients. Murine alveolar macrophages (AMs) were cultured *ex vivo* with/without human MSC-EVs before adoptive transfer to LPS-injured mice.

**Measurements and Main Results:** MSCs suppressed cytokine production, increased M2 macrophage marker expression and augmented phagocytic capacity of human MDMs stimulated with LPS or ARDS BALF. These effects were partially mediated by CD44-expressing EVs. Adoptive transfer of AMs pre-treated with MSC-EVs reduced inflammation and lung injury in LPS-injured mice. Inhibition of oxidative phosphorylation in MDMs prevented the modulatory effects of MSCs. Generating dysfunctional mitochondria in MSCs using rhodamine-6G pre-treatment also abrogated these effects.

**Conclusions:** In the ARDS environment, MSCs promote an anti-inflammatory and highly phagocytic macrophage phenotype through EV-mediated mitochondrial transfer. MSC-induced changes in macrophage phenotype critically depend on enhancement of macrophage oxidative phosphorylation. AMs treated with MSC-derived EVs ameliorate lung injury *in vivo*.

**Word count: 244**

**Keywords:** acute respiratory distress syndrome; extracellular vesicles; mesenchymal stromal cells; macrophages; mitochondria

## Introduction

Acute Respiratory Distress Syndrome (ARDS) is the leading cause of mortality and morbidity in the critically ill. While mortality rates have fallen with modifications in mechanical ventilation, they remain as high as 25-40% and no effective pharmacological treatments are available<sup>(1-3)</sup>. ARDS results from multiple causes, pneumonia and sepsis being the most common and devastating. Uncontrolled alveolar inflammation is the hallmark of this disease. Alveolar macrophages (AMs) provide defence against respiratory pathogens and orchestrate inflammatory responses in the distal respiratory tract. Macrophages are polarized by environmental cues and adopt different phenotypes. M1 pro-inflammatory and M2 anti-inflammatory phenotypes are associated with the acute and resolving phases of inflammation in ARDS respectively<sup>(4, 5)</sup>. Mesenchymal Stromal Cells (MSCs) are increasingly recognized as a promising candidate therapy for ARDS<sup>(6)</sup>. We and others have reported that MSCs improve survival, reduce inflammation and enhance bacterial clearance in pre-clinical models of lung injury<sup>(7-14)</sup>. Secretion of paracrine factors, modulation of host cells via secretion of extracellular vesicles (EVs) and mitochondrial transfer were shown to be important for the therapeutic effects of MSCs in these studies. These data have lent support to phase I/II clinical trials testing MSC administration to ARDS patients<sup>(15, 16)</sup>. At present however, there are no potency assays in place to aid in the selection of MSC donors before their administration to patients<sup>(17)</sup> and despite rapid progression of MSCs to clinical trials, a complete understanding of the mechanisms of MSC immunomodulatory effects remains elusive.

MSCs have the capacity to transfer mitochondria to alveolar epithelium enhancing bioenergetics and mitigating lung injury<sup>(9, 18)</sup>. Our group previously demonstrated that

MSCs transfer mitochondria to human macrophages via tunnelling nanotubes which enhanced their phagocytic capacity and facilitated MSC antimicrobial effects in murine *E. coli* pneumonia<sup>(19)</sup>. Importantly, depletion of AMs abrogated MSC protective effects in this model suggesting that AMs are key cellular mediators of MSC effects. While contact-dependent mechanisms such as these are important, a large body of evidence suggests that MSC therapeutic effects are mediated by paracrine factors<sup>(20-22)</sup>. Recently EVs have emerged as an important component of the MSC secretory repertoire. MSC-EVs may contain a diverse cargo including proteins, mRNAs, miRNAs and mitochondria, the functional effects of which remain largely unknown<sup>(9, 23-25)</sup>. MSC-derived EVs alone are capable of recapitulating many of the beneficial effects of MSC whole cell therapy<sup>(25-28)</sup>. Phinney *et al* observed the transfer of MSC mitochondria and miRNAs via EVs to human macrophages in a model of silicosis and found that mitochondria enhanced macrophage bioenergetics while miRNAs suppressed Toll-like receptor signalling<sup>(24)</sup>. They observed that normoxia (21% oxygen) induced oxidative stress thereby promoting mitophagy in MSCs. Ghanta *et al* then went on to demonstrate the importance of autophagy in maintaining healthy mitochondrial function and promoting survival in MSCs during oxidative stress<sup>(29)</sup>. The influence of mitochondrial transfer on macrophage phenotype however, has not been studied extensively. Vats *et al* showed the importance of mitochondrial oxidative phosphorylation in the induction of IL-4-induced anti-inflammatory M2 macrophage polarization<sup>(30)</sup>. Indeed, glucose metabolism is intrinsically linked to macrophage activation state with M1 pro-inflammatory macrophages utilizing glycolysis<sup>(31)</sup>. In this study, we sought to characterize the effects of MSCs on human macrophage polarization in the *in vitro* models of ARDS. We tested the hypothesis that mitochondrial transfer from MSCs to

macrophages via EVs modulates macrophage function through promotion of oxidative phosphorylation. Some of the results of these studies have been previously reported in the form of an abstract<sup>(32-35)</sup>.

## Methods

See the online supplement for detailed methods.

### *Human Bone Marrow-Derived Mesenchymal Stromal Cells (MSCs)*

MSCs were acquired from the Texas A&M Health Science Centre College of Medicine, Institute for Regenerative Medicine (Temple, Texas, USA), a National Institutes of Health repository. These cells fulfil all criteria set by the International Society of Cellular Therapy for definition of MSCs<sup>(36)</sup>. Multiple MSC donors were used for experiment throughout this study.

### *Human Monocyte-Derived Macrophage (MDM) and MSC Non-Contact Co-Culture*

Monocytes were isolated from donor buffy coats as previously described<sup>(37)</sup>. Buffy coats were obtained from the Northern Ireland Blood Transfusion Service. Ethical approval was granted by the School Research Ethics Committee of Queen's University Belfast. Monocytes were differentiated into macrophages for 7 days in the presence of 10ng/mL GM-CSF (R&D Systems). Macrophages were cultured with MSCs in the Transwell system at a ratio of 1:5 or with MSC conditioned medium (CM). Cells were stimulated with lipopolysaccharide (LPS) (*Escherichia coli* O111:B4, List Biological Laboratories) at 10ng/mL or 30% bronchoalveolar lavage fluid (BALF) pooled from nine patients with ARDS diluted in RPMI-1640 1% FBS for 24 or 72 hours. Ethical approval for use of patient samples for research was obtained from the

Office for Research Ethics Committee Northern Ireland. In additional experiments, MSC-CM was pre-treated with CD44 neutralizing antibody (BD Biosciences) to assess the importance of EV uptake.

#### *Isolation of MSC-derived EVs*

MSC-EVs were isolated as previously described<sup>(27)</sup>. Briefly,  $15 \times 10^6$  cells were cultured in media supplemented with EV-depleted serum for 48 hours. CM was then collected and centrifuged at 10,000g for 20 mins to remove cells and debris, followed by centrifugation at 100,000g for 2 hours to isolate EVs. EVs were resuspended in 2mL of media ( $7.5 \times 10^6$  cells/mL). Flow cytometry was used for characterization of EVs using the FACSCanto II flow cytometer and FACSDiva software. Analysis was performed using FlowJo v7 software.

#### *In vivo LPS-induced lung injury model*

C57BL/6 male mice (8- to 10-weeks old; Harland Institute, UK) were used. Animals were maintained in the Biological Services Unit at Queen's University Belfast. Experiments were sanctioned and approved by the UK Home Office and Queen's University Belfast Ethical Review Committee. Mice were anaesthetized by isoflurane inhalation and 20mg/kg of LPS was instilled intranasally. 4 hours after injury, AMs from *ex vivo* culture were given intranasally ( $2.5 \times 10^5$  AMs/mouse) following xylazine/ketamine anaesthetic. 24 hours after injury, mice were culled and BALF was taken for analysis.

#### *Mitochondrial transfer and functional studies*

For assessment of mitochondrial transfer MSC and MDM mitochondria were pre-stained with MitoTracker® Deep Red and Green respectively (Thermofisher). CM was taken from pre-stained MSCs and added to MDMs for 24 hours to allow EV



uptake, mitochondrial transfer was visualized using the EVOS® FL Auto Imaging System (Life Technologies). For functional studies, MDM mitochondrial function was inhibited with oligomycin at 3µg/mL; MSC mitochondria were inhibited by pre-treatment for 48 hours with 1µg/mL of rhodamine-6G (both Sigma Aldrich), which both target ATP synthase<sup>(38, 39)</sup>. Mitochondrial respiration was assessed using the Seahorse XF Cell Mito Stress Test kit and XF<sup>e</sup>96 Extracellular Flux Analyzer. Analysis was performed using Wave v2.2 software (all Agilent Technologies).

### *Statistical analysis*

Analysis was performed using GraphPad Prism 5 software. Experiments for each MDM donor were performed at least in triplicate; the average of three technical replicates were taken as a single data point for each donor and pooled together for statistical analysis. Pooled data were presented as the mean with standard deviation. For parametric data, Student's t-test or one-way ANOVA with post-hoc analysis using Bonferroni's selected comparisons was performed. For non-parametric data, Kruskal-Wallis with post-hoc analysis using Dunn's selected comparisons was used. Statistical significance was regarded as  $p < 0.05$ .

## **Results**

### *MSCs induce an unconventional M2-like phenotype in MDMs with increased phagocytic capacity in the presence of E. coli LPS*

LPS stimulation increased MDM secretion of pro-inflammatory cytokines TNF-α and IL-8. MSCs significantly diminished TNF-α and IL-8 production by 58±8% and 30±15% respectively (**Figure 1A, B**). **Table 1** summarizes the effects of MSC on

MDM production of cytokines and chemokines associated with M1 and M2 macrophage polarization.

The M1 cytokines IFN $\gamma$ , IL-1 $\beta$ , IL-12p70 and IL-17 as well as the M2 anti-inflammatory cytokine IL-10 were not detectable in these co-cultures. Levels of the M1 cytokines IL-23, the M1 chemokine CCL5, the M2 cytokine IL-1ra and the M2 chemokine CCL17 were unaffected by MSCs. Levels of the M2 chemokines CCL18 (68 $\pm$ 21% reduction) and CCL22 (79 $\pm$ 12% reduction) were also diminished with MSCs although the reduction in CCL18 levels was not significant.

In this study we have investigated expression of an array of markers previously shown to be suitable for human macrophages (**Table 2**). Among those only expression of CD206 (marker for M2 polarization<sup>(40)</sup>) demonstrated differential regulation by LPS and MSC; MDM expression of CD206 was significantly increased by MSCs both in the presence (40 $\pm$ 16% increase) or absence (25 $\pm$ 12% increase) of LPS. (**Figure 1C**). Notably, expression of CD163 (an established M2 marker<sup>(41)</sup>) although detectable was not affected by any of the stimulations. MSCs significantly increased the proportion of phagocytic MDMs in the presence of LPS by 2.9-fold compared to LPS alone (**Figure 1D**). In contrast, fibroblasts cell control studies demonstrated that although fibroblasts diminished TNF- $\alpha$  secretion by MDMs, they did not influence IL-8 levels or CD206 expression, indicating that the observed effects were specific to MSC (**Figure E1**).

#### *MSCs modulate MDM phenotype and function in the BALF taken from ARDS patients*

To more closely mimic ARDS environment, MDMs were co-cultured with MSC in 30% BALF of ARDS patients or healthy volunteers. ARDS BALF resulted in a sharp up-

regulation of TNF- $\alpha$  secretion which MSCs significantly reduced by  $53\pm 16\%$  (**Figure 2A**). MSCs reduced IL-8 levels in the presence of ARDS BALF, however this did not reach statistical significance (**Figure E2**). Consistently, MSCs were able to significantly increase the proportion of MDMs expressing the M2 marker CD206 (**Figure 2B**). In the presence of ARDS BALF, MSCs were able to double the proportion of phagocytic MDMs (**Figure 2C**).

*MSC-derived EVs expressing CD44 are partially responsible for the MSC anti-inflammatory effect and enhanced phagocytosis*

Flow cytometric analysis demonstrated that MSC-CM contains a population of particles of less than  $4\mu\text{m}$  in diameter (herein referred to as EVs). This is consistent with the literature showing that MSC-EVs range from  $<100\text{nm}$  to  $1000\text{nm}$ <sup>(24, 27)</sup>. Further characterization demonstrated that EVs were  $>90\%$  positive for MSC cell membrane,  $>90\%$  negative for annexin V and propidium iodide staining, indicating minimal contamination with cell debris or apoptotic bodies and importantly EVs demonstrated uniform expression of CD44 ( $>98\%$  positive) (**Figure 3A**).

Pre-incubation of MSC-CM with anti-CD44 antibody, but not with IgG, partially abrogated the effect on MDM TNF- $\alpha$  secretion (suppression of  $70\pm 19\%$  reduced to  $43\pm 15\%$  after CD44 neutralization). Importantly, anti-CD44 antibody did not alter TNF- $\alpha$  secretion by MDMs without presence of MSC-CM (**Figure 3B**). As before, MSC-CM significantly increased MDM phagocytic activity in the presence of LPS. The percentage of phagocytic MDMs was increased by  $28\pm 5\%$  and MDMs phagocytic index (determined by Median Fluorescent Intensity (MFI)) was increased 9-fold, compare to LPS stimulation alone. Anti-CD44 but not IgG completely reversed

these effects. Antibodies given to MDMs in the absence of MSC-CM had no influence on phagocytic activity (**Figure 3C**).

*Adoptive transfer of murine AMs treated by MSC-EVs confer protection in LPS-induced lung injury*

In order to test if modulation of macrophages by MSC-EVs has therapeutic effect *in vivo*, AMs were isolated from C57BL/6 mice, treated *ex vivo* with MSC-EVs for 48 hours and adoptively transferred intranasally into mice which were challenged with LPS to induce lung injury. Intranasal instillation of LPS to mice resulted in lung injury at 24 hours as evidenced by increased inflammatory cell infiltration and protein content in the BALF. Treatment of mice with MSC-EV-treated AMs 4 hours after LPS challenge reduced total cell counts in the BALF by  $45\pm 13\%$ , absolute neutrophil counts by  $61\pm 10\%$ , BALF TNF- $\alpha$  levels by  $40\pm 15\%$  and BALF protein by  $52\pm 6\%$  compared to LPS-injured mice receiving vehicle control (**Figure 4A**). Importantly, treatment of mice with *ex vivo*-cultured AMs which were not treated with MSC-EVs had no effect (**Figure 4B**). Cytospin preparations of BALF demonstrate substantial inflammatory cell recruitment to the alveolar compartment consisting predominantly of neutrophils in the LPS-injured group which is reduced by administration of EV-treated AMs (**Figure 4C**).

*MSC-EVs transfer functional mitochondria to MDMs enhancing macrophage oxidative phosphorylation*

Flow cytometric analysis of MSC-CM demonstrates that 25% of MSC-EVs detectable by this method are positive for mitochondria (**Figure 5A**). Mitochondrial transfer from MSCs to MDMs was visualized by fluorescence microscopy. MSCs were pre-stained

with MitoTracker® Red FM, CM collected and added to MDMs which had been pre-stained with MitoTracker® Green FM and Hoechst nuclear stain (blue). At 24 hours, there is evidence of MSC mitochondria-containing EVs adhering to the MDMs (red staining, white arrows) and co-localization of MSC mitochondria into the MDM mitochondrial network (yellow staining, yellow arrow) (**Figure 5B**).

To specifically inhibit mitochondrial respiration in MSCs, MSCs were pre-treated with rhodamine-6G which irreversibly binds to ATP synthase<sup>(38)</sup>. Rhodamine treatment abrogated MSC mitochondrial respiration while enhancing non-mitochondrial respiration (**Figure E3, A**); importantly the capacity of MSCs to secrete paracrine factors (Ang-1, IL-8) was not significantly affected (**Figure E3, B, C**), ruling out non-specific effects of rhodamine on MSC capacity to secrete soluble factors. Rhodamine did not induce cell death in MSCs after 48h (**Figure E3, D**). MSC-CM enhanced mitochondrial respiration capacity in MDMs compared to MDMs stimulated with LPS alone. CM from rhodamine pre-treated MSCs had no effect on these parameters (**Figure 5C**).

*Transfer of mitochondria-containing MSC-EVs is responsible for MSC modulation of MDMs through enhanced oxidative phosphorylation*

The ATP synthase inhibitor oligomycin drastically increased the MDM TNF- $\alpha$  response to LPS (3.8-fold). Oligomycin treatment had no cytotoxic effects on MDMs after 24 hours (**Figure E4**). Importantly, the addition of oligomycin completely prevented the anti-inflammatory effect of MSC-CM (**Figure 6A**). Similarly, oligomycin completely reversed the effect of MSC-CM on MDM phagocytosis (**Figure 6B**). These results provide evidence for direct involvement of mitochondrial oxidative metabolism in MSC-CM-mediated modulation of macrophage function. CM from

MSCs pre-treated with rhodamine-6G lose the capacity to reduce LPS-induced TNF- $\alpha$  production, enhance the phagocytic capacity of MDMs and upregulate expression of M2 marker CD206 (**Figure 6C-E**). In aggregate, these data demonstrate that MSCs modulate primary human macrophages through EV-mediated transfer of functional mitochondria which enhance macrophage oxidative phosphorylation.

## Discussion

The following conclusions can be drawn from this study: 1) MSCs reprogram human macrophages in the presence of *E. coli* LPS and ARDS patient BALF via paracrine mechanisms; the MSC-induced macrophage phenotype is characterized by a dampened inflammatory cytokine secretory profile, increased expression of the M2 marker CD206 and enhanced phagocytic capacity (**Figures 1 and 2**); 2) MSC-EVs expressing CD44 are partially responsible for the suppression of MDM TNF- $\alpha$  production and promoting phagocytosis (**Figure 3**); 3) MSC-EV-treated murine AMs protect from endotoxin-induced lung injury *in vivo* (**Figure 4**); 4) the transfer of functional mitochondria in EVs is responsible for MSC anti-inflammatory and phagocytosis enhancing effects on macrophages in the inflammatory environment through the promotion of oxidative phosphorylation (**Figures 5, 6**).

Phinney *et al* previously showed that MSCs donate their mitochondria to human macrophages under oxidative stress via EV-mediated transport thereby enhancing their bioenergetics<sup>(24)</sup>. Our group has recently shown that mitochondrial transport via tunnelling nanotubes from MSCs to MDMs was important for enhancing phagocytic capacity<sup>(19)</sup>. The current study shows for the first time that the transfer of mitochondria via MSC-EVs promotes phagocytosis and suppresses pro-inflammatory

cytokine secretion by human macrophages. This mitochondrial transfer was associated with increased oxidative phosphorylation in MDMs which was necessary for these modulatory effects. Moreover, this work demonstrates that modulation of AMs by MSC-derived EVs is sufficient to mitigate lung injury *in vivo*.

We first sought to characterize human macrophage modulation by MSC in the *in vitro* models of inflammation investigating cytokine and chemokine secretion, expression of characteristic surface markers and phagocytic activity. MSCs were able to reduce MDM production of two major pro-inflammatory cytokines associated with ARDS severity TNF- $\alpha$  and IL-8<sup>(42, 43)</sup> in the presence of *E. coli* LPS (**Figure 1A, B**). This corroborates previous reports showing that MSCs are able to suppress secretion of pro-inflammatory cytokines by macrophages<sup>(40)</sup>. Other tested M1 and M2-associated cytokines and chemokines were undetectable or unaffected by MSCs although the M2 chemokine CCL22 was reduced (**Table 1**). Notably, IL-10 was not detectable in these cultures; this is in controversy with a number of reports describing upregulation of IL-10 production by macrophages that are cultured with MSCs<sup>(40, 44)</sup>. This discrepancy may be explained by the inherent variability of the immunoregulatory capacity of MSC donors. Moreover, macrophages differentiated in GM-CSF, like in the present study, have been shown to express low levels of IL-10 after LPS treatment<sup>(45)</sup>.

MSCs consistently upregulated expression of the key M2 macrophage marker CD206 in the presence of LPS, while other markers of M1 and M2 macrophages were unaffected (**Figure 1C and Table 2**). This is in agreement with previous reports describing MSC induction of M2-type macrophages characterized by CD206 expression<sup>(40, 46)</sup>. The lack of effect on other M1/M2 markers is perhaps a reflection of the lack of well-defined markers to unambiguously define human macrophage

activation state<sup>(47)</sup>. It could also be explained by the different methods for monocyte differentiation and macrophage stimulation; in our studies we used GM-CSF as a differentiation factor to model AMs<sup>(48, 49)</sup>. GM-CSF also promotes an M1-like phenotype, more closely mimicking activated AMs which would be present in the alveoli of ARDS patients. MSCs were able to increase the proportion of phagocytic MDMs in the presence of LPS (**Figure 1D**). This adds to the body of literature supporting the phagocytosis enhancing effects of MSCs on both macrophages and monocytes<sup>(8, 10, 19, 27)</sup>.

This study demonstrates for the first time that MSCs are capable of modulating MDM phenotype and function in the presence of BALF taken from ARDS patients. MSCs were able to reduce TNF- $\alpha$  secretion, increase CD206 expression and promote phagocytosis in MDMs exposed to ARDS BALF (**Figure 2**), effectively mimicking the distal lung microenvironment of these patients. Induction of a less pro-inflammatory AM which exhibits increased phagocytic capacity may improve outcomes in ARDS which is classically associated with a rampant inflammatory response and substantial bacterial burden such as sepsis or pneumonia-induced ARDS<sup>(1)</sup>. These data add valuable clinical relevance to these *in vitro* studies. Importantly, we have shown previously that MSCs are able to migrate into the alveolar spaces even when given intravenously<sup>(19)</sup>, therefore it is plausible that MSC may produce similar effects on AMs of patients. Additionally, the capacity of MSCs to induce polarization towards M2-type monocytes/macrophages, identified by CD206 expression, may serve as a biomarker for MSC efficacy in clinical samples. EVs have emerged as a major contributor to the therapeutic effects of MSCs in lung injury, capable of recapitulating many of the effects of the whole cell therapy<sup>(25-27)</sup>. CD44 expression on MSC-EVs was shown to be necessary for their uptake and therapeutic effect on target cells<sup>(27,</sup>



<sup>50</sup>). In the current study, prevention of EV uptake by MDMs using anti-CD44 neutralizing antibody abrogated the ability of MSC-CM to reduce MDM TNF- $\alpha$  secretion and enhance phagocytosis (**Figure 3B, C**). This suggests that MSC-EVs are primarily responsible for MSC modulation of MDM function.

To confirm the importance of MSC-EV uptake by AMs in lung injury *in vivo*, endotoxin-injured mice were given murine AMs, pre-treated with MSC-EVs *ex vivo*. AMs exposed to MSC-EVs but not untreated AMs were able to significantly reduce the extent of lung injury after 24 hours, demonstrated by reduced inflammatory cell recruitment and decreased BALF protein levels (**Figure 4**). These data corroborate our previous findings where clodronate-based depletion of AMs abrogated the beneficial effects of MSCs in lung injury *in vivo*, demonstrating that AMs are cellular mediators of the MSC effect<sup>(19)</sup>. Importantly, these new data not only highlight AMs as key cellular targets of MSC-EVs but also emphasize an essential role of AMs in mitigating lung injury. This is the first study to report the efficacy of adoptive transfer of AMs in endotoxin-induced lung injury. Adoptive transfer of macrophages has also been investigated in colitis and airway hyper-responsiveness with promising results<sup>(51, 52)</sup>. While not true AMs, Litvack *et al* have also shown that stem cell-derived alveolar-like macrophages improve bacterial and neutrophil clearance in another model of lung injury<sup>(49)</sup> but further study is required to determine the safety and feasibility of these treatment modalities. Moreover, these data contribute to the growing body of literature which suggests the potential of MSC-EVs as a therapy in place of MSCs<sup>(25-27)</sup>. While there have been no reports of MSC-induced neoplasia to date, EVs cannot themselves transform to form tumours reducing the potential risk associated with cell-based therapy. It has been shown that different methods of isolation can influence vesicle yield, purity and contents<sup>(53)</sup>. Obtaining a sufficient

quantity of EVs from MSCs for their effective use in the clinic also presents a significant challenge; a consensus must be reached on the optimal culture conditions and EV isolation protocols to standardize this process.

Our group and others have previously shown the transfer of mitochondria from MSCs to human macrophages via EVs<sup>(19, 24)</sup>. In this study, MSC-EV-mediated mitochondrial transport to MDMs was visualized with fluorescence microscopy (**Figure 5B**). The treatment of MDMs with MSC-CM resulted in enhanced bioenergetics in the macrophages evidenced by increased basal mitochondrial respiration as well as ATP turnover. MSC-CM taken from rhodamine-6G-pre-treated MSCs with dysfunctional mitochondria were unable to affect these parameters (**Figure 5C**). These findings are in line with previous reports of improved bioenergetics in cells receiving MSC mitochondria including macrophages, alveolar epithelial cells and vascular endothelial cells<sup>(9, 18, 19, 24, 54)</sup>. To confirm that production of soluble mediators was sustained in rhodamine-6G-treated MSCs, angiopoietin-1 and IL-8 levels were quantified after LPS treatment with or without rhodamine-6G pre-treatment. While diminished, these paracrine factors were still produced at high levels suggesting that paracrine secretion by MSCs was not greatly affected by inhibition of their mitochondrial function (**Figure E3, B, C**).

M1 pro-inflammatory macrophages have been shown to utilize glycolytic metabolism<sup>(31)</sup>. Vats *et al* highlighted the importance of mitochondrial oxidative phosphorylation in the induction of M2-type macrophages through IL-4 signalling. They observed that mitochondrial inhibition in macrophages blocked the anti-inflammatory effects of IL-4<sup>(30)</sup>. In the present study, addition of the mitochondrial inhibitor oligomycin prevented the suppression of TNF- $\alpha$  and the enhancement of phagocytosis in macrophages by MSC-CM, demonstrating that the MSC effect is

critically dependent on oxidative phosphorylation (**Figure 6A, B**). We hypothesized that mitochondrial transfer via MSC-EVs was responsible for both enhancing MDM phagocytosis and suppressing pro-inflammatory cytokine production through the promotion of oxidative phosphorylation. Indeed, MSC-CM taken from rhodamine-6G-pre-treated-MSCs with dysfunctional mitochondria had no effect on TNF- $\alpha$  production, phagocytosis or CD206 expression in LPS-treated MDMs (**Figure 6C-E**).

There are limitations to this study. The BALF experiments used ARDS BALF diluted to 30% by volume; while exposing the MDMs to all of the constituents of the ARDS microenvironment, this will effectively reduce the concentrations of the stimuli it contains. The endotoxin-induced lung injury model was relatively mild<sup>(14, 25)</sup>, however the primary aim of the *in vivo* experiments was to provide a proof of principle that AMs are important cellular mediators of MSC-EV effects using a gain-of-function approach. Additionally, we did not investigate the mechanisms by which MSC-EV-treated murine AMs are protective in lung injury.

In conclusion, this study demonstrates that in the inflammatory environment of ARDS, MSCs modulate human macrophages towards decreased production of pro-inflammatory cytokines, increased expression of the M2 phenotype marker CD206 and enhanced phagocytic capacity. MSC-EVs carrying mitochondria are responsible for these effects through the promotion of oxidative phosphorylation in macrophages, uncovering a novel mechanism of modulation of macrophage polarization. Moreover, this work suggests that changes in AMs induced by MSC-derived EVs are sufficient to elicit protection in lung injury *in vivo*. The ability of MSCs to promote CD206 expression in human macrophages may serve as a biomarker of MSC efficacy in ARDS patients.

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## Figure legends

**Figure 1.** Human mesenchymal stromal cell (MSC) modulation of human monocyte-derived macrophage (MDM) phenotype and function. (A) MSCs in non-contact co-culture reduce the production of TNF- $\alpha$  by MDMs after 24h of lipopolysaccharide (LPS) treatment. One-way ANOVA with Bonferroni's post-hoc test (n=5 per group). (B) IL-8 production by MDMs was reduced by MSC co-culture after LPS treatment. One-way ANOVA with Bonferroni's post-hoc test (n=4 per group). (C) MSC co-culture increases the percentage of MDMs expressing CD206 on their surface in the absence (i) or presence (ii) of LPS. Unpaired t-test (n=3 per group) and one-way ANOVA with Bonferroni's post-hoc test (n=4 per group) respectively. (D) MSCs increase the proportion of MDMs which had phagocytosed *E. coli* pHrodo® fluorescent BioParticles® after LPS. One-way ANOVA with Bonferroni's post-hoc test (n=5 per group). Data are presented as mean  $\pm$  standard deviation. \*p<0.05, \*\*p<0.01.

**Figure 2.** Human mesenchymal stromal cells (MSCs) modulate human monocyte-derived macrophages (MDMs) in the presence of Acute Respiratory Distress Syndrome (ARDS) patient bronchoalveolar lavage fluid (BALF). (A) MSCs reduce the production of TNF- $\alpha$  by MDMs treated with 30% ARDS BALF for 24 hours. Kruskal-Wallis with Dunn's post hoc test (HV BALF n=3, other groups n=5). (B) MSCs increase the proportion of MDMs expressing the M2 macrophage surface marker CD206 after 72 hours. One-way ANOVA with Bonferroni's post-hoc test (n=3 all groups). (C) MSCs increase the proportion of phagocytic MDMs in the presence of ARDS BALF. One-way ANOVA with Bonferroni's post-hoc test (n=4 all groups). Data

are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ . HV BALF - healthy volunteer BALF, BALF - ARDS BALF.

**Figure 3.** CD44-expressing extracellular vesicles (EVs) from human mesenchymal stromal cells (MSCs) are partially responsible for their modulatory effects. Flow cytometry demonstrates that (A)(i) MSC-derived EVs are smaller in diameter than latex beads of 4 $\mu$ m diameter and (ii) are positive for CD44 expression on their surface. (B) Pre-incubation of MSC-CM with anti-CD44 neutralizing antibody partially reversed MSC suppression of TNF- $\alpha$  secretion by MDMs (n=4 all groups) and completely prevented MSC enhancement of (C)(i) the proportion of phagocytic MDMs as well as (ii) their phagocytic index (n=5 all groups). One-way ANOVA with Bonferroni's post-hoc test. Data are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ .

**Figure 4.** Human mesenchymal stromal cell (MSC) extracellular vesicle (EV)-treated alveolar macrophages (AMs) reduce lipopolysaccharide (LPS)-induced lung injury. Treatment of LPS-injured mice with EV-treated AMs reduced total cell counts and neutrophilic cell counts as well as the amount of TNF- $\alpha$  and protein (A) in the bronchoalveolar lavage fluid (BALF). One-way ANOVA with Bonferroni's post-hoc test (A, B and D - n=4 PBS, n=5 LPS, n=3 LPS+EV-AM). Student's unpaired t-test (C - n=3 LPS, n=3 LPS+EV-AM). Untreated AMs had no effect on total or neutrophil cell counts and had no effect on BALF TNF- $\alpha$  or protein levels (B). One-way ANOVA with Bonferroni's post-hoc test (F, G and I - n=4 PBS, n=4 LPS, n=5 LPS+unt-AM). Student's unpaired t-test (H - n=5 LPS, n=5 LPS+unt-AM). Cytospins of BALF

preparations demonstrate inflammatory cell recruitment to the airspaces after LPS injury and the reduced cell numbers after treatment with EV-treated AMs but not untreated AMs (images taken at 20x magnification) (*E*). Data are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Figure 5.** Human mesenchymal stromal cell (MSC)-derived extracellular vesicles (EVs) transfer functional mitochondria to human monocyte-derived macrophages (MDMs) which enhances macrophage oxidative phosphorylation. (*A*) Flow cytometry of culture medium taken from MitoTracker® Red pre-stained MSCs shows that 25% of EVs are positive for mitochondria. (*B*) EVs contained in MSC culture medium (CM) transfer mitochondria (red) to MDMs (MitoTracker® Green) (white arrows). MSC mitochondria are integrated into the MDM mitochondrial network (yellow, yellow arrows) (images at 20x magnification). (*C*) Treatment of MDMs with MSC-CM resulted in an increase in basal mitochondrial respiration (*i*) as well as mitochondrial ATP turnover (*iii*) determined by the Seahorse metabolic analyzer. The increase in maximal mitochondrial respiration did not reach statistical significance (*ii*). Kruskal-Wallis with Dunn's post-hoc test ( $n=4$  all groups). Data are presented as mean  $\pm$  standard deviation. \* $p < 0.05$ . OCR - oxygen consumption rate.

**Figure 6.** Mitochondrial transfer via human mesenchymal stromal cell (MSC)-derived extracellular vesicles (EVs) modulates human monocyte-derived macrophage (MDM) function through enhancement of macrophage oxidative phosphorylation. (*A*) Addition of oligomycin (reversible ATP synthase inhibitor) prevented the suppression of TNF- $\alpha$  by MSC culture medium (CM) ( $n=5$  all groups but DMSO  $n=4$ ). (*B*)

Oligomycin also prevented MSC-CM enhancement of the proportion of phagocytic MDMs (*i*) and phagocytic index (*ii*) (n=7 all groups). Pre-treatment of MSCs with rhodamine-6G (irreversible ATP synthase inhibitor) similarly reversed MSC-CM capacity to suppress TNF- $\alpha$  production (*C*) (n=4 all groups), enhance the phagocytic capacity of MDMs (*D*) (n=4 all groups) as well as promoting M2 marker CD206 expression (*E*) (n=5 all groups). All one-way ANOVA with Bonferroni's post-hoc test. Data are presented as mean  $\pm$  standard deviation. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Table 1: Effect of MSCs\* on MDM† cytokine and chemokine secretion after LPS‡ stimulation**

<i>Analyte</i>		<i>Concentration (pg/mL) ± standard deviation</i>		<i>P-value</i>
		<i>LPS</i>	<i>LPS+MSC</i>	
<i>M1-associated</i>	IFN $\gamma$	ND	ND	-
	IL-1 $\beta$	ND	ND	-
	IL-8	9,155 ± 1,790	6,410 ± 1,676	<0.05
	IL-12p70	ND	ND	-
	IL-17	ND	ND	-
	IL-23	37,856 ± 27,577	23,858 ± 17,544	0.361
	TNF- $\alpha$	2,465 ± 1,103	1,038 ± 207	<0.05
	CCL5	9,571 ± 8,032	5,885 ± 4,392	0.404
<i>M2-associated</i>	IL-1ra	120,143 ± 34,335	110,304 ± 27,258	0.627
	IL-10	ND	ND	-
	CCL17	678 ± 607	341 ± 228	0.589
	CCL18	904 ± 604	292 ± 190	0.056
	CCL22	106,211 ± 67,673	22,486 ± 13,267	<0.05

Abbreviations: \*MSCs – human mesenchymal stromal cells, †MDM – human monocyte-derived macrophage, ‡LPS – lipopolysaccharide

Table 2: Effect of MSCs\* on macrophage marker expression

		Marker	Expression (%)		P-value
			-MSC	+MSC	
M1-associated	No LPS†	CD40	70.2 ± 10.8	85.3 ± 7.6	0.114
		CD54	97.1 ± 2.5	98.6 ± 0.8	0.686
	LPS	CD40	95.8 ± 1.4	97.7 ± 1.4	0.147
		CD54	97.9 ± 0.8	98.8 ± 1.1	0.200
M2-associated	No LPS	CD163	11.6 ± 14.5	28.0 ± 9.0	0.090
		CD206	7.0 ± 0.8	31.8 ± 11.7	<0.05
	LPS	CD163	0.2 ± 0.1	0.2 ± 0.2	0.686
		CD206	23.3 ± 9.5	63.5 ± 16.3	<0.05

Abbreviations: \*MSCs – human mesenchymal stromal cells, †LPS – lipopolysaccharide



Fig 1

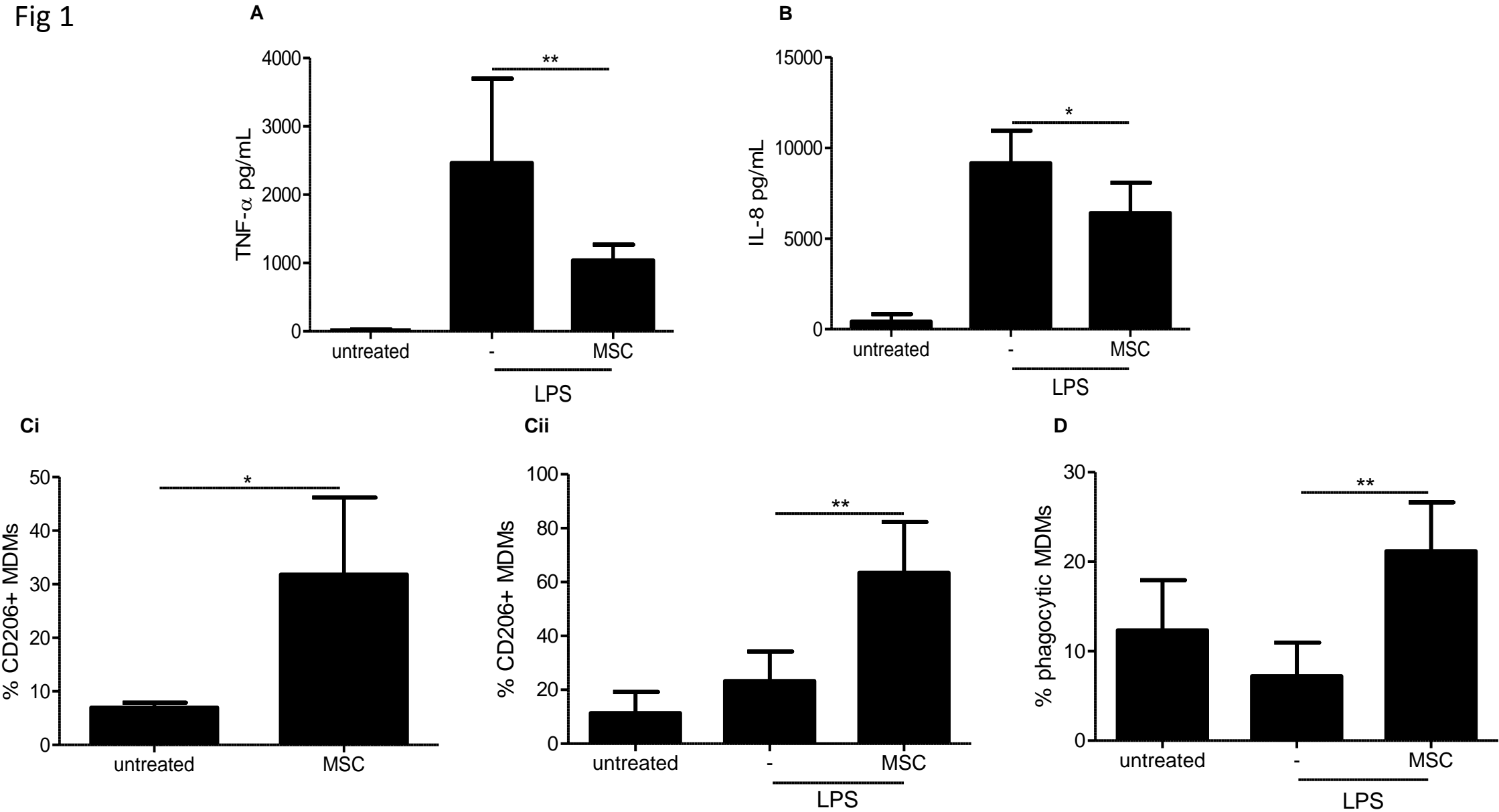
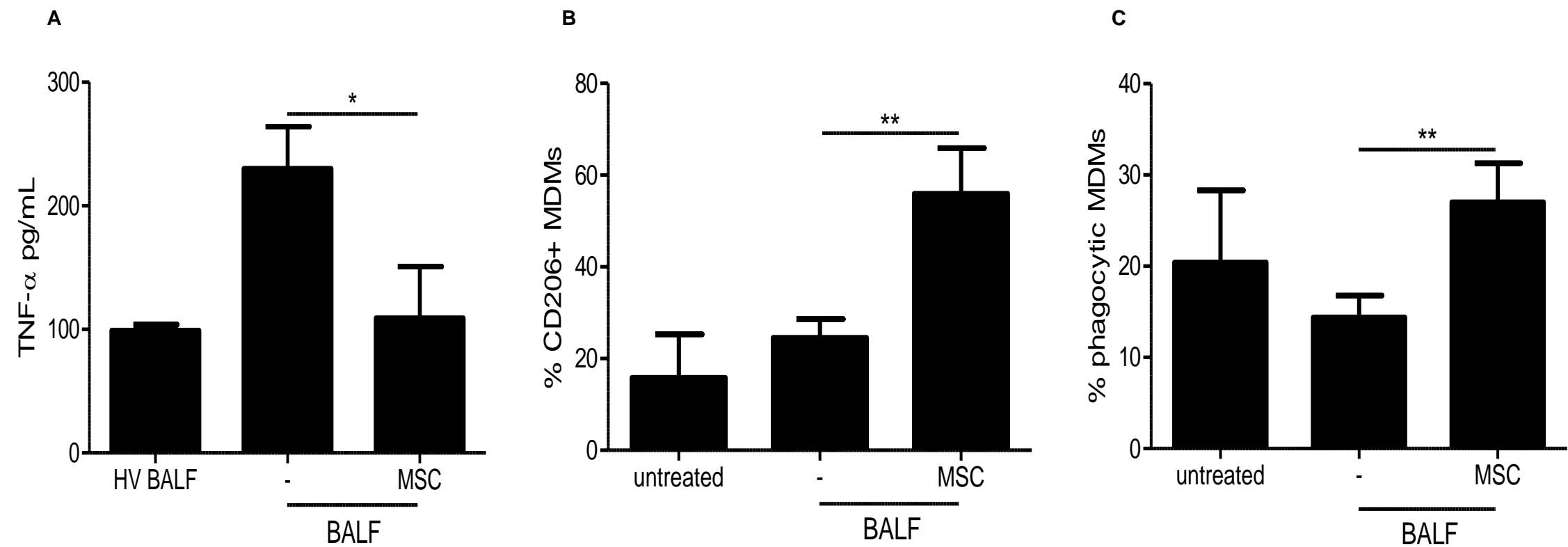


Fig 2



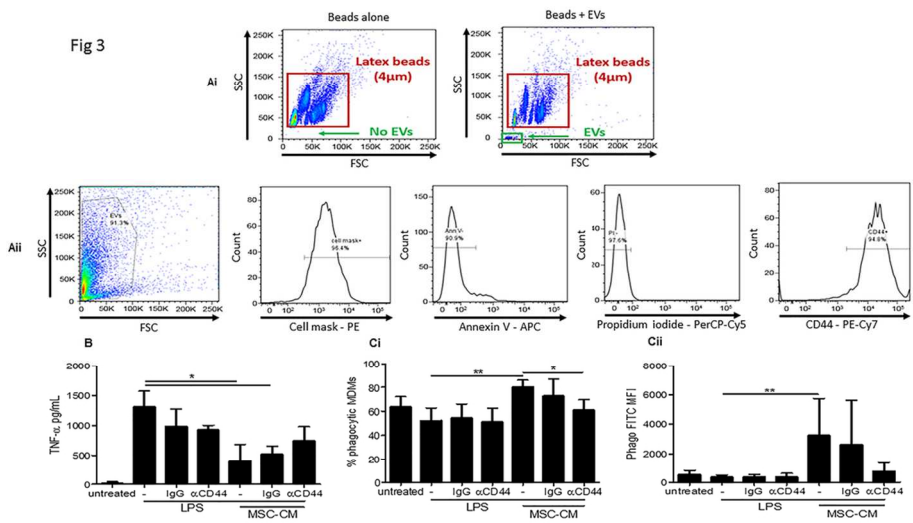


Figure 3

54x30mm (600 x 600 DPI)

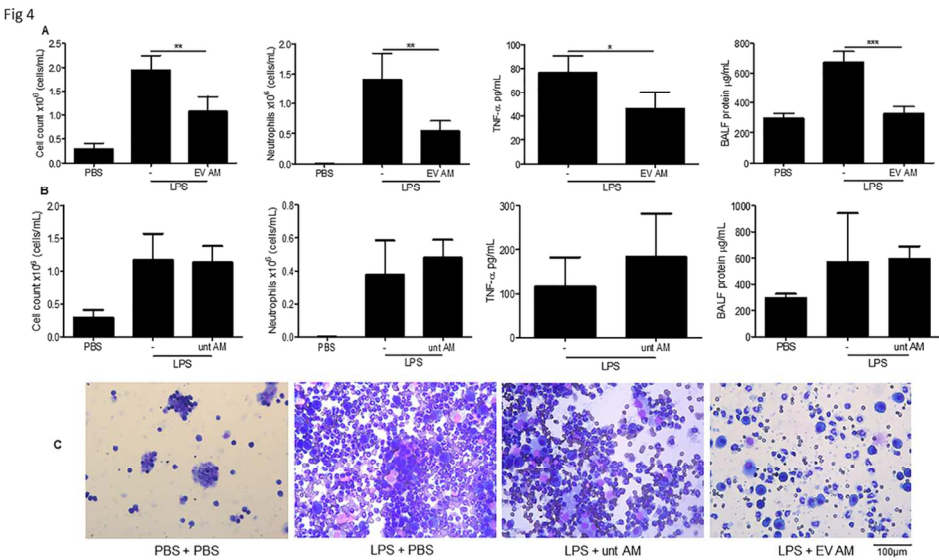


Figure 4  
54x30mm (600 x 600 DPI)

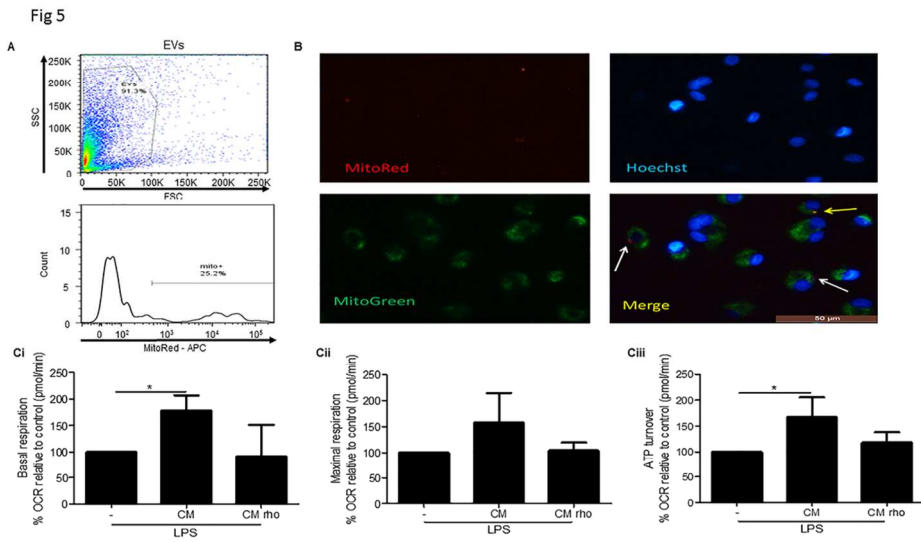
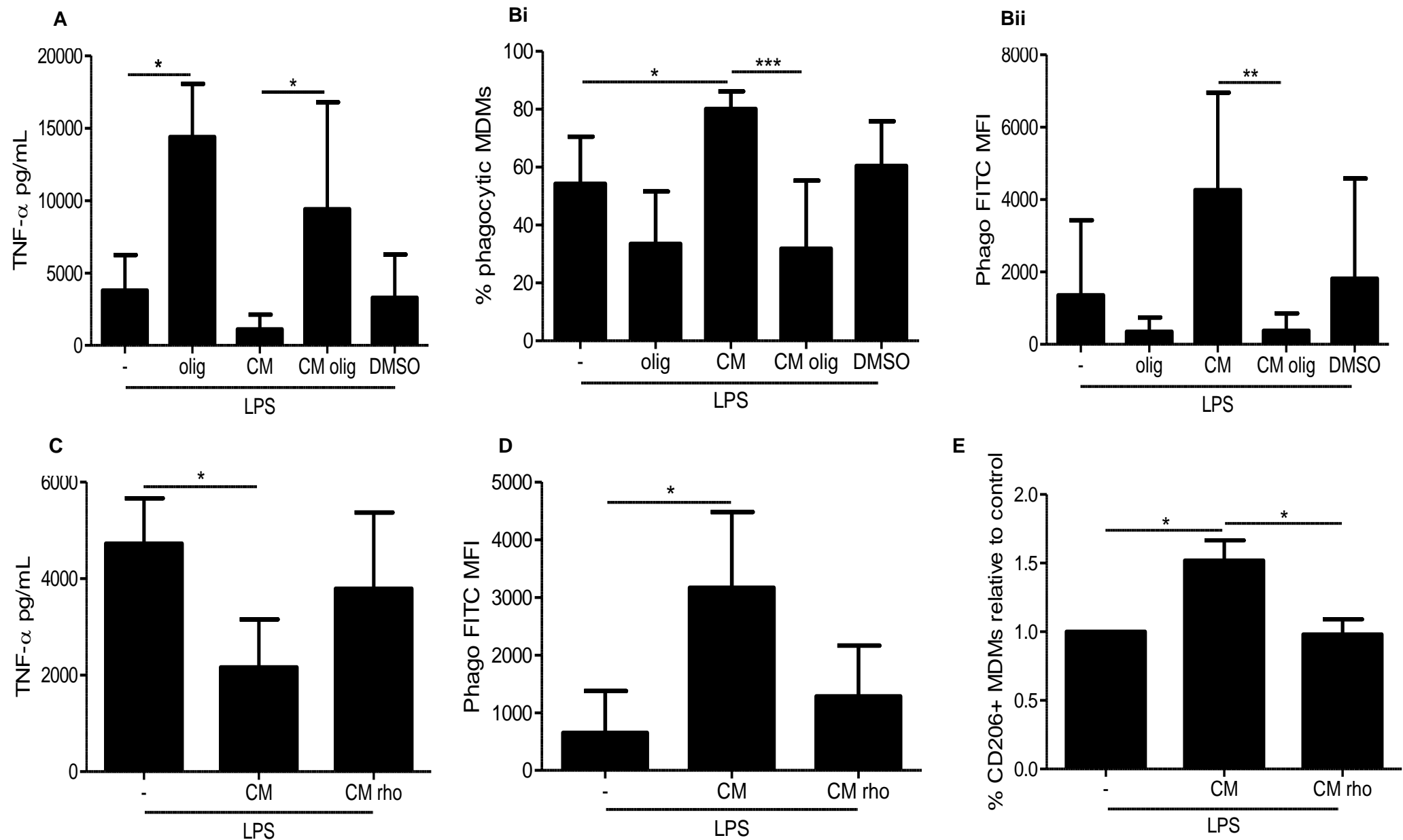


Figure 5  
54x30mm (600 x 600 DPI)

Fig 6



# **Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer**

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Krasnodembskaya A

## **ONLINE DATA SUPPLEMENT**

## MATERIALS AND METHODS

### Culture of human bone marrow-derived MSCs

Bone marrow-derived MSCs were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White through a grant from NCRR of the National Institute of Health (NIH), Grant # P40RR017447. These MSCs were extensively characterized by the supplier testing for plastic adherence, expression of key mesenchymal markers and absence of haematopoietic markers, as well as differentiation capacity for a number of mesenchymal lineages. These assessments fulfil the criteria put forward by the International Society for Cellular Therapy for characterization and identification of MSCs. MSCs were cultured under standard tissue culture conditions; 37°C, 5% CO<sub>2</sub> and 21% O<sub>2</sub>. MSCs were grown in T175 culture flasks using  $\alpha$ -Minimal Essential Medium ( $\alpha$ -MEM) supplemented with 16.5% heat-inactivated foetal bovine serum (FBS), 1% L-glutamine and 50 $\mu$ g/mL penicillin-streptomycin (PS) (all from Gibco, Thermofisher) and fed every three days. 20mL of media was used to ensure complete coverage of the T175 flask. MSCs were grown to 70% confluence before being passaged. MSCs were lifted from culture by first aspirating the media, washing once with deionised PBS (DPBS) and adding trypsin-EDTA (both from Gibco, Thermofisher) diluted in DPBS to a concentration of 0.05% for 3-4 minutes at 37°C and 5% CO<sub>2</sub>. The flasks were then tapped forcefully to encourage detachment. Once detached, an equal volume of  $\alpha$ -MEM<sub>16.5%FBS+PS</sub> was added to neutralize the trypsin. The cell solution was then centrifuged at 1200rpm for 5 mins and the supernatant aspirated. The pellet was resuspended in a volume of media equating to 1mL of  $\alpha$ -MEM<sub>16.5%FBS+PS</sub> per T175 flask of cells before performing a cell count.



Cryovials containing 1 million MSCs were taken from liquid nitrogen storage and quickly thawed at 37°C in a water bath for 2 minutes. The 1mL cell suspension was then added to 10mL of pre-warmed  $\alpha$ -MEM<sub>16.5%FBS+PS</sub>. The cells were centrifuged at 1200rpm for 5 mins and the supernatant was decanted off, removing the dimethylsulfoxide (DMSO) (Sigma Aldrich). The pellet was resuspended in 1mL of media before being added to a pre-warmed T175 flask and returned to the incubator. If the MSCs were for expansion, before 24 hours the MSCs were lifted using trypsin as described earlier, counted and seeded across T175 flasks at a cell density of 60 cells/cm<sup>2</sup>. When the cells reached 70% confluence they were detached, pooled, counted and diluted in media to a concentration of 2 million cells/mL. The cell solution was then diluted 1:1 using freezing medium consisting of  $\alpha$ -MEM<sub>50%FBS+PS</sub> and DMSO at a ratio of 9:1, bringing the cell concentration to 1 million cells/mL. 1mL of the cell solution/freezing solution mixture was then added to each cryovial, placed in a freezing container filled with isopropanol and stored overnight at -80°C. The next morning the cryovials are transferred to liquid nitrogen for long term storage.

### **Human monocyte-derived macrophage (MDM) culture**

Buffy coats from single donors were obtained from the Northern Ireland Blood Transfusion Service (NIBTS); these buffy coats are what remains after blood donations are processed to extract the majority of the red cell fraction. Ethical approval for the use of these buffy coats was acquired through the Queen's University Belfast Research Ethics Committee (Ref: 14/35 Title: Investigating regulation of inflammation and repair in the Acute Respiratory Distress Syndrome

using blood cells extracted from buffy coat). This leaves a white cell enriched sample, which is depleted of platelets and a large proportion of red cells. Diluted the buffy coat (approximately 40-65mL) in Hank's Balanced Salt Solution (HBSS) minus  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Gibco, Thermofisher) up to a volume of 140mL. Added 15mL of Ficoll Paque Premium (GE Healthcare) to a 50mL falcon tube and then slowly layered the blood mixture on top at a 45° angle, being careful not to mix the two layers, to produce four 50mL falcons. Centrifuged at 480g for 20 minutes at 20°C with the brakes off. This separates the blood components by density centrifugation and produces a white layer containing lymphocytes and monocytes. Used a Pasteur pipette to extract the white cell layer from the tubes taking care not to remove red cells. Pooled the cells from each tube into one falcon before adding HBSS up to 50mL. Centrifuged at 1200rpm for 5 minutes at 4°C. Aspirated the supernatant, taking care not to lose the cell pellet. This is done to remove any residual Ficoll that would have been extracted along with the cells. Repeated the wash step with HBSS two more times. Resuspended the cell pellet in 20mL of Roswell Park Memorial Institute (RPMI) (Gibco, Thermofisher) media supplemented with 1% heat-inactivated FBS and 50µg/mL penicillin-streptomycin and prepared an aliquot with a 10x dilution of cells in media. Added 20uL of this to a haemocytometer and place in the incubator for 5 minutes to allow monocytes to adhere. Performed a cell count using a light microscope and haemocytometer, adherent monocytes will appear cloudy/translucent. Seeded the monocytes at the desired density in  $\text{RPMI}_{1\% \text{FBS+PS}}$  for at least 2 hours to allow adherence of the monocytes. All other contaminating cells, e.g. erythrocytes and lymphocytes, will not adhere. Monocytes were seeded at 300,000 per well in 24-well plates and 1,000,000 per well in 6-well plates. Aspirated the  $\text{RPMI}_{1\% \text{FBS+PS}}$  from the adherent

monocytes and washed twice with HBSS to remove contaminating cells. Added RPMI<sub>10%FBS+PS</sub> further supplemented with 10ng/mL of GM-CSF (R&D Systems, Biotechne). GM-CSF will differentiate the monocytes into macrophages over the 6 to 7-day incubation.

### **Non-contact co-culture of human MSCs and human MDMs**

After 6-7 days of differentiation MDM co-culture with MSCs could begin. Before beginning the experiment, MDMs were serum starved in unsupplemented RPMI for 2 hours. MSCs used in experiments were passage 3-5. MSCs would be suspended in culture above the MDMs using 0.4µm porous hanging cell culture transwell inserts (Merck Millipore). These required pre-soaking in media for at least 20 mins prior to adding the cells to facilitate cell adherence and to allow co-culture without contact. For all co-culture experiments, MSCs were prepared at a 5:1 ratio of MDMs to MSCs. Conditions were prepared in RPMI<sub>1%FBS+PS</sub> with any additional stimuli at a volume of 500µL for 24-well plates and 2mL for 6-well plates.

### ***In vitro* stimulation experiments**

To generate an inflammatory environment, *E. coli* LPS O111:B4 (List Biological Laboratories) was added to the MDMs and MSCs at a working concentration of 10ng/mL in RPMI<sub>1%FBS+PS</sub>. In later experiments, to mimic the microenvironment of the alveolar compartment of patients with ARDS, BALF from ARDS patients was used for stimulation in place of LPS. These BALF samples were those taken from the clinical trial performed by Craig *et al* and published in 2011, where they

investigated the use of simvastatin as a treatment for ARDS<sup>(394)</sup>. BALF samples used for these stimulation experiments were baseline samples taken prior to any intervention. BALF from nine of these patients were pooled to generate a stock and the pooled sample was then diluted to 30% in RPMI<sub>1%FBS+PS</sub> before stimulation. When possible healthy volunteer BALF was included to demonstrate whether it is ARDS patient BALF or BALF in general that is responsible for the observed effects. Healthy volunteer samples were taken from the study published by Shyamsundar *et al* in 2014 investigating the effects of KGF in mild, LPS-induced lung injury<sup>(395)</sup>. The healthy volunteer BALF samples used for these stimulations were from individuals who had received neither LPS nor KGF.

### **Enzyme-linked Immunosorbent Assay (ELISA)**

Human TNF- $\alpha$  and IL-8 levels in cell supernatants were quantified using ELISA duoset kits (R&D Systems, Biotechne). Exact working concentrations of the kits constituents may be found in the manufacturer's instructions. Capture antibody for the cytokine of interest was prepared in 1xPBS (Gibco, Thermofisher) to the desired concentration and 100 $\mu$ L of this solution was added to each well of a Maxisorp 96-well ELISA plate (Nunc). The plate was then sealed and incubated overnight at room temperature. The capture antibody was aspirated and the wells washed using wash buffer (1xPBS with 0.05% Tween-20 (Sigma Aldrich)) three times, blotting against clean paper towels after each wash. Care was taken to remove as much liquid as possible after the final wash when blotting. Non-specific binding was prevented by blocking the plates with 300 $\mu$ L of reagent diluent per well (1xPBS with 1% bovine serum albumin (BSA) (Sigma Aldrich)) for at least 1

hour. Again, the plates were washed three times with wash buffer and blotted before addition of the cytokine standards and supernatant samples. A two-fold dilution series was used to prepare a range of standard concentrations and then 100µL of these and the samples were added to each well for a 2 hour incubation. The wells were washed three times and 100µL of detection antibody prepared in reagent diluent was added for another 2 hour incubation. After another three washes, 100µL of streptavidin-horse radish peroxidase diluted 1:40 in reagent diluent was added to each well, and incubated for 20 mins protected from light. A final three washes was performed and 100µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Life technologies) was added and incubated for 20 mins, or until the standards had suitably developed, protected from light. 50µL of 2M sulphuric acid stop solution was added to each well and the plate gently tapped to ensure mixing. The ELISA was then immediately analyzed using the Versamax spectrophotometer set to read at 450nm and 540nm wavelength. A subtraction of the wavelength absorbance readings at 540nm was taken from the readings at 450nm for correction. 4-parameter standard curves were produced using Softmax Pro v2.6 and concentrations of samples were extrapolated.

## **Bioplex**

The measurement of multiple other cytokines and chemokines was performed using a custom Luminex Performance Assay Multiplex kit (R&D Systems, Biotechne). The analytes for this assay were the cytokines IL-1 $\beta$ , IL-1ra, IL-10, IL-12, IL-23 and IFN $\gamma$  and the chemokines CCL5, CCL17, CCL18 and CCL22. The assay was performed according to manufacturer's instructions. The kit was used

along with a Luminex analyzer that uses a dual laser flow-based sorting and detection platform. Each analyte's antibody is provided already coated onto coloured microparticles. Standards are provided for the analytes and a 3-fold dilution series is used to generate a range. 50 $\mu$ L of either the standard/microparticle or sample/microparticle mixtures were added to the plate provided. The plate was then incubated at room temperature for 3 hours with gentle agitation on a shaker before washing with 100 $\mu$ L of the wash buffer provided with the kit. Wash buffer was then removed with a vacuum manifold dispenser which siphons the liquid through filters on the base of the plate. This wash step was repeated two more times. 50 $\mu$ L of streptavidin-phycoerythrin was added to each well and incubated for 30 mins protected from light on a shaker. Three more washes were performed and a fourth wash was allowed to sit for two minutes before aspiration. There are lasers present in the apparatus which are specific to each of the colour-coded microparticles which should be conjugated to the antibody-analyte complexes. Another laser will excite any of the streptavidin-phycoerythrin fluorophore which has now bound and is present in levels proportional to the amount of analyte in the samples. The plate is then analyzed and sample analyte concentrations were deduced by plotting the standard concentrations against the median fluorescence intensity (MFI) using a 5-parameter logistic fit.

### **Flow cytometry**

Flow cytometry was used for analysis of macrophage surface markers, phagocytosis assays and EV characterization. After the experimental time point

was reached inserts and supernatants were removed from the MDMs and the cells were washed with PBS. 1mL of flow buffer (PBS 5%FBS) was added to the cells and they were gently scraped from the well plates using a cell scraper. The cell solution was then added to 5mL polystyrene flow tubes (Sarstedt, Fisher Scientific) and centrifuged at 350g for 5 mins at 4°C to pellet the cells. The tubes were then decanted to remove the flow buffer, leaving 200µL in the tube. The cells were resuspended in 0.5mL of flow buffer and 20µL of human FcR binding inhibitor (eBioscience) was added. The cells were incubated on ice for 20 mins to block non-specific binding. Following this, the antibodies (**see Table E1**) would be added at the necessary dilution and incubated for 30 mins on ice, protected from light. For each experiment a number of controls were included, an unstained control, isotypes for each fluorophore in use and single stain controls for each fluorophore. For experimental groups, all antibodies of interest were added to each tube. After the antibody incubation, 3mL of flow buffer was added to each tube and the cells were centrifuged at the same settings as earlier. The supernatant was aspirated and the wash step repeated. After the second wash, 200µL was left in each flow tube and the samples were run. Samples were processed using the FACSCantoII flow cytometer and FACSDiva software (BD Biosciences). Where necessary, compensation between fluorophores was performed on the FACSDiva software by using single stain controls. Data was then analyzed using FlowJo v7 software (Treestar).

Table E1: Antibodies used for macrophage markers

Cell surface marker	Fluorochrome conjugate	Manufacturer
Anti-human CD40	FITC	eBioscience
Anti-human CD54	PE	eBioscience
Anti-human CD163	APC	eBioscience
Anti-human CD206	PE-Cyanine7	eBioscience

**Flow cytometric phagocytosis assay**

To determine the phagocytic capacity of MDMs with or without co-culture of MSCs, fluorescently labelled pHrodo<sup>®</sup> green *E. coli* BioParticles<sup>®</sup> (Thermofisher) were used. Briefly, after 24 hours of co-culture, the supernatant is removed and the MDMs are washed twice with PBS. *E. coli* particles were then suspended in RPMI<sub>1%FBS+PS</sub> with 10µL of stock per 1mL of media (provides particles at a 20:1 ratio particles to MDMs). *E. coli* bioparticles were then added to MDMs in a total volume of 2mL for 6-well plates. The plates were then centrifuged at 350g for 5 mins at 4°C to ensure contact between the MDMs and the particles. MDMs were incubated at 37°C and 5%CO<sub>2</sub> for 90 minutes, protected from light, to allow phagocytosis to occur. A number of wells are not treated with particles and serve as an unstained control. As recommended by the manufacturer, a number of wells



were treated with the particles but incubated on ice as a control. These cells should not be capable of phagocytosis and any signals detected in these wells should be subtracted as background. After the incubation, the supernatant was aspirated, and the cells were washed twice in PBS. A final 1mL of PBS was added to each well and the MDMs are gently scraped. The cell solution was centrifuged at 350g 4°C for 5 mins and the supernatant aspirated before adding 200µL of PBS to the tube for resuspension. The samples were then analyzed by flow cytometry.

### **CD44 blockage experiments**

To determine if MSC-EVs were responsible for MSC effects on MDMs, blockage studies were performed. MSC-CM was generated and treated with an antibody for human CD44 (mouse anti-human CD44, BD Biosciences) or isotype control (mouse IgG<sub>2B</sub>, R&D Systems, Biotechne) to a final concentration of 3µg/mL or given no antibody. They were incubated for 30mins at 37°C with gentle mixing to allow the antibody to bind any EVs present in the CM and then added on to serum starved MDMs with 10ng/mL of LPS as described earlier. Importantly CD44 is also expressed on macrophages, so in order to control for any effects of this antibody on MDMs directly, additional groups were included where MDMs were treated with the antibodies without MSC-CM. After 24 hours of stimulation, supernatants were extracted for cytokine analysis.

### **Generation of EV-free FBS**

For EV work, media was prepared with heat-inactivated FBS which was free of all contaminating EVs. FBS was taken in 40mL centrifuge tubes (Hitachi) and

ultracentrifuged at 100,000g using the Himac CP100WX ultracentrifuge (Hitachi) for 3 hours at 4°C with a P28S rotor (Hitachi). The supernatant was then taken and used to supplement either  $\alpha$ MEM or RPMI for treating the MSCs.

### **Isolation of MSC-EVs**

MSCs were grown to around 60-70% confluence under standard conditions before having their media replaced with 15mL of EV-depleted  $\alpha$ MEM<sub>16.5%FBS+PS</sub> (for microvesicle characterization experiments) or RPMI<sub>1%FBS+PS</sub> (for addition to MDMs after preparation). The MSCs were incubated for 48 hours at 37°C and 5%CO<sub>2</sub> and the culture medium was collected. Firstly, the medium was centrifuged at 10,000g at 4°C for 20 mins to remove cells and cell debris. The supernatant was then ultracentrifuged at 100,000g for 2 hours at 4°C and the supernatant was aspirated. The pellet of EVs was resuspended in PBS to fill the 40mL tubes before centrifuging again at the same settings. This acts as a wash to minimize contamination with protein aggregates. The PBS supernatant is then discarded and the EV pellet is finally resuspended for downstream processing.

### **Quantification of MSC-EVs**

In a pilot experiment, MSC-EVs were isolated as described above and the concentration of RNA and protein was assessed using the Nanodrop 2000 (ThermoFisher). Briefly, MSC-EVs were washed and resuspended in 100 $\mu$ L of PBS. 1 $\mu$ L of this resuspension was added to the Nanodrop 2000. The RNA concentration was found to be 0.47ng/ $\mu$ L corresponding to 47ng of RNA from the isolation. The EV isolation had a protein concentration of 1470 $\mu$ g/mL corresponding to 147 $\mu$ g of protein in the isolation.

### Characterization of MSC-EVs by flow cytometry

To provide an indication of the size of MSC-EVs, MSC-CM was processed by flow cytometry with the addition of aldehyde/sulphate latex beads (ThermoFisher) which have a diameter of 4µm. The latex beads provide a reference point to compare with the EVs in FSC vs SSC plots. MSCs were grown to confluence and stained with a number of different reagents. Stains used for the characterization of EVs are listed in **Table E2**. MSCs were treated with human FcR binding inhibitor for 20 minutes at 4°C to prevent non-specific binding. They were stained with CellMask™ Orange (cell membrane), propidium iodide (necrosis), Annexin V (apoptosis), anti-human CD44 (or isotype control) and MitoTracker® Deep Red FM for 45 minutes at 4 °C in the dark.

*Table E2: Stains used for the characterisation of MSC-EVs*

Stain	Fluorochrome conjugate	Manufacturer
CellMask™ Orange	PE	ThermoFisher
Propidium iodide	PerCP-Cy5.5	Sigma Aldrich
Annexin V	Pacific Blue	Biolegend
Anti-human CD44	PE-Cy7	eBioscience
MitoTracker® Deep Red FM	APC	ThermoFisher

After the incubation, MSCs were washed three times in PBS and EV-free αMEM<sub>16.5%FBS+PS</sub> was used to prepare CM. After 24 hours, CM was lifted and

processed immediately by flow cytometry (FACSCantoII flow cytometer and FACSDiva software). Unstained MSC-CM was processed first in order to determine the necessary FSC and SSC voltage settings required to detect the EVs. Single stain controls were then tested, as well as the isotype for anti-CD44, to set gates for these stains and to perform the necessary compensation. The gating strategy was as follows: EVs were gated for by FSC vs SSC; cell membrane positive events were selected using CellMask™; events which were negative for propidium iodide (necrosis) staining were then gated for; Annexin V staining (apoptosis) was excluded. This final gate was then used to determine the extent of CD44 expression by EVs and the proportion of these EVs which contained mitochondria. To provide some information on the size of these EVs, latex beads with a diameter of 4µm were processed by flow cytometry first separately and then in combination with MSC-CM. FSC vs SSC plots of the latex beads provided a reference point to compare with the size of the EVs.

### **Animal studies**

C57BL/6 male mice (8 to 10 weeks old; Harland Institute, UK) were used. Animals were maintained in the Biological Services Unit (BSU) at Queen's University Belfast. Experiments were sanctioned and approved by the UK Home Office and Queen's University Belfast Ethical Review Committee.

### **Bronchoalveolar lavage (BAL)**

Mice were culled with intraperitoneal (IP) injection of 300µL of xylazine/ketamine mixture. The skin of the upper body and neck was cut open and the trachea was

exposed. The connective tissue surrounding it was cut away and a length of string was placed in behind the trachea. A small hole was cut in the anterior wall of the trachea and a severed 21 gauge needle was introduced through the opening. The string was then tied around the trachea anchoring the needle in place and 1mL of PBS was instilled into the lungs. The PBS was flushed in and lifted out 7-10 times to ensure thorough sampling of the bronchoalveolar compartment and to isolate as much AMs as possible.

### ***Ex vivo* culture of mouse AMs**

A 20 $\mu$ L aliquot of the BALF samples were mixed with 20 $\mu$ L of trypan blue and a cell count was performed. Murine AMs were seeded into 6-well plates at 3 million per well in the PBS they were extracted in but then spiked with FBS to generate PBS<sub>1%FBS</sub>. The AMs were given 4 hours to adhere before they were gently washed with PBS and then treated with MSC-EVs suspended in RPMI<sub>1%FBS</sub> or media alone. For EV-treated AMs, each well was treated with the EVs generated from 10 million MSCs over 48 hours. AMs were cultured *ex vivo* for 48 hours before they were detached using 1mL of Accutase® cell detachment reagent (Innovative Cell Technologies) incubated for 5 mins at 37°C. Any cells remaining attached were gently dislodged with a cell scraper. The cell viability was determined using trypan blue staining and another cell count was performed. The cells were then centrifuged at 5000g for 5 mins, the supernatant aspirated and the AMs resuspended in PBS such that 35 $\mu$ L of solution contained  $2.5 \times 10^5$  AMs.

### **LPS model of lung injury**

Mice were first anaesthetized by isoflurane inhalation for 1-2 minutes individually. Mice were given 0.8µg of LPS per gram of body weight. *E. coli* LPS was administered intranasally (IN) diluted in PBS at a volume of 35µL. Control mice were given PBS alone. Mice were observed until they recovered from the isoflurane. 4 hours after the LPS instillation, mice were anaesthetized once again using 100µL of xylazine/ketamine injected using a 25 gauge needle IP. Mice were then treated with either vehicle control (PBS), *ex vivo*-cultured MSC-EV-treated AMs or untreated AMs ( $2.5 \times 10^5$  AMs per mouse) IN in a volume of 35µL. Mice were observed until recovery and returned to holding. 24 hours after LPS instillation, mice were culled and subjected to BAL to be used for cell counts, protein quantification and cytokine analysis.

### **Total cell counts**

BALF samples were first diluted 2-fold in PBS into fresh eppendorfs, 20µL of this was mixed with 20µL of trypan blue and then 10µL of this mixture was added to each side of a Countess® cell counting chamber slide (Invitrogen, Thermofisher). Total cell counts were carried out using the EVE™ automated cell counter (NanoEnTek, VWR). Two counts were taken for each BALF sample and dilutions were accounted for in the final cell counts expressed as cells/mL.

### **Cytospin preparation, imaging and neutrophil counts**

After taking the aliquot for total cell counts, BALF samples were centrifuged at 5000g for 5 mins to remove the cells from suspension. Supernatants were lifted and placed in a fresh eppendorf then stored at -20°C for future processing. The cell pellet was resuspended in 300µL of PBS and centrifuged at 10,000rpm for 5 mins using the StatSpin Cytofuge®2 (Beckman Coulter, VWR) onto microscope slides. Slides were given 2 hours to dry and then stained using the Speedy Diff kit (Clin-tech). Slides were given another hour to dry before imaging using the Leica Epifluorescence DM5500 microscope at 20x magnification. Enough images were taken of each slide in order to count a total of 400 cells. Cells were counted using ImageJ software and the percentage of neutrophils was determined. Using this percentage, total neutrophil counts were calculated from the total cell counts taken earlier.

### **RNA isolation**

RNA isolation was carried out using the RNeasy mini kit (Qiagen) as per manufacturer's instructions. After the experiment, MDMs were washed in PBS and then 350µL of RLT lysis buffer supplemented with β-mercaptoethanol (inhibits the action of RNase enzymes, 10µL per 1mL of lysis buffer) was added to each well. To ensure complete lysis of cells, scraped the base of each well using a fresh cell scraper and then collected the lysates in a sterile eppendorf. Samples were homogenized by passing the solutions through a 25 gauge microneedle 10 times. Further processing of RNA was maintained at 4°C throughout. An equal volume of

70% ethanol was added to the lysates and the solutions were mixed before placing in the spin columns and centrifuging at full speed for 30 seconds. The flow through was discarded and 700 $\mu$ L of RW1 buffer was added. Columns were centrifuged again and the flow through was aspirated. Next 500 $\mu$ L of RPE buffer, supplemented with ethanol, was added and again centrifuged. This RPE buffer wash was repeated one more time and then the collection tube holding the spin column replaced with a fresh one before centrifuging once again. This step is used to ensure that all ethanol has been removed from the columns, as ethanol interferes with the final RNA elution step. The spin columns are then placed in a final collection eppendorf and 30 $\mu$ L of RNase free water is placed in the column. A final centrifugation extracts a pure, concentrated RNA sample.

### **Quantification of RNA**

Total RNA purity and concentration was determined using the Nanodrop 2000. The software was first initialized and then a baseline blank was established by placing 1 $\mu$ L of RNase free water on the platform of the device. After setting a blank, 1 $\mu$ L of the RNA samples were then added and analyzed. Between sample additions, the platform was cleaned using a paper towel soaked with 70% ethanol to prevent any sample mixing. Two readings were taken for each sample and an average was calculated. A 260/280 purity ratio of 1.8 or higher was considered pure enough for downstream processing. Before proceeding to reverse transcription, the concentrations of all samples were first normalized to the lowest sample concentration of that batch using RNase free water.



### Real-time qualitative PCR

RT-PCR was carried out using TaqMan universal PCR master mix along with TaqMan primer assay (mRNA) (Thermofisher). PCR reactions were prepared in a 96 well plate with a total volume of 20 $\mu$ L per reaction:

Reagent	Volume (per reaction)
Master mix (2X)	10 $\mu$ L
Primer assay (20X)	1 $\mu$ L
cDNA diluted in ddH <sub>2</sub> O	9 $\mu$ L

For every PCR plate prepared, a no template control and a no reverse transcription control was included. The no template control contained master mix, the primers of interest and only ddH<sub>2</sub>O. The no reverse transcription control contained master mix, primers and an equal amount of RNA which had not been converted to cDNA. Each sample was added in duplicate for each assay being run. Once the plate had been loaded, the wells were sealed with strip caps, the plate was mixed on a shaker for 30 seconds and then centrifuged at 1500rpm for 2 mins to collect the solutions to the base of each well. The plate was loaded into a Stratagene Mx3005P PCR device (Agilent Technologies) and the RT-PCR reaction was setup like so:

Step number	Condition settings	Number of cycles
1	50°C for 2 mins	1
2	95°C for 10 mins	1
3	95°C for 15 secs 60°C for 1 min	50

**Studying mitochondrial transfer**

MSCs were grown to confluence and pre-stained with MitoTracker® Deep Red for 45 mins at 37°C before being washed three times with PBS. CM was prepared from pre-stained MSCs for 24 hours. MDMs had their nuclei pre-stained with Hoechst 33342 (Sigma Aldrich) and their mitochondria pre-stained with MitoTracker® Green (Thermofisher) for 45 mins at 37°C. MDMs were also washed with PBS before treating with MSC-CM and incubating the MDMs for 24 hours to allow EV uptake. MDMs were washed once more with PBS and treated with fully supplemented RPMI. Fluorescence imaging of live MDMs was performed using the EVOS® FL Auto Imaging System (Life technologies) at 20x magnification.

**Generating dysfunctional mitochondria**

Loss-of-function experiments were performed to assess the importance of mitochondrial transfer from MSCs in their effects on MDMs. In order to produce dysfunctional mitochondria, MSCs were treated with rhodamine-6G (Sigma Aldrich) at a concentration of 1µg/mL for 48 hours at 37°C as previously described<sup>(399)</sup>. Because mitochondrial respiration would be blocked by this

treatment, MSCs had their media supplemented to support glycolysis. Standard  $\alpha$ -MEM<sub>16.5%FBS+PS</sub> was supplemented further with uridine to produce a concentration of 50 $\mu$ g/mL and sodium pyruvate to a concentration of 2.5mM. After 48 hours, MSCs were washed three times with PBS before generating CM for experiment. To further study the importance of mitochondrial respiration in MDM function, MDMs were treated with 3 $\mu$ g/mL of oligomycin which was prepared in DMSO and added to RPMI<sub>1%FBS+PS</sub>. DMSO alone was used as a vehicle control.

### **Assessing mitochondrial respiration**

To determine the efficiency of rhodamine-6G as a mitochondrial inhibitor and to assess the effect of MSC-CM on MDM mitochondrial activity, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) was used. MSCs or MDMs were seeded into XF 96-well tissue culture plates at densities of 10,000 and 30,000 cells per well respectively and allowed to adhere overnight. The cells were then treated with the various conditions for 24-48 hours depending on the experiment. During this incubation a cartridge plate provided with the kit, which contained the probes for measuring oxygen consumption, was soaked in XF calibrant solution and left to incubate at 37°C and 0% CO<sub>2</sub>. Cells were washed twice with XF basal medium which had been supplemented with 10mM glucose, 1mM pyruvate and 2mM glutamine. 180 $\mu$ L of XF basal medium was added to each well and the cells were placed in the incubator at 37°C and 0% CO<sub>2</sub> for 1 hour. Meanwhile the oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone/antimycin-A inhibitors were prepared as per the manufacturer's instructions. The probe cartridge was collected from the incubator and the inhibitors were added to specific ports of these cartridges. 20 $\mu$ L of oligomycin was added to port A, 22 $\mu$ L of FCCP was added to port B and 25 $\mu$ L of

rotenone/antimycin-A was added to port C for each well. These inhibitors would be sequentially injected into the cell solution during the Mito Stress Test. The loaded cartridge plate was then placed in the XF<sup>e</sup>96 Extracellular Flux Analyser (Agilent Technologies) for calibration. After calibration, the XF 96-well tissue culture plate containing the cells was added to the machine and the Mito Stress Test was performed. Oxygen consumption rate readouts produced by the assay were analyzed using the Wave software (Version 2.2) (Agilent Technologies).

### **Lactate dehydrogenase (LDH) assay**

For pilot ARDS patient BALF, oligomycin and rhodamine-6G stimulation experiments, cytotoxicity assays were performed on the hMDMs or hMSCs using the Cytotoxicity Detection Kit (LDH) (Life Sciences, Roche). After treatment the supernatants were lifted and cell debris was removed by centrifugation at 500G for 10 mins. 50µL of each sample was added in duplicate to wells of a Maxisorp 96-well ELISA plate (Nunc). Assay mixture was prepared by mixing 250µL of the diaphorase/NAD<sup>+</sup> catalyst with 11.25mL of the iodotetrazolium chloride/sodium lactate dye solution. 50µL of the assay mixture was added to each well on top of the samples and mixed on a shaker for 1 minute. The assay mixture is pink and will turn red as the assay develops. The 96-well plate was then incubated at room temperature for 30 mins before being read by a spectrophotometer at 490nm. A number of controls are included for this assay; a background control is 100µL of the assay mixture and is used as a blank which is subtracted from all other values. There is an untreated control of hMDMs cultured in RPMI<sub>1%FBS+PS</sub> alone to determine basal LDH release. Then there is the cell death control which consisted of RPMI<sub>1%FBS+PS</sub> with 2% Triton-X detergent (Sigma Aldrich) to induce total cell death. Cell death was calculated as a percentage relative to the cell death control.

Absorbance readings of the background control were subtracted from all other readings. Then the following calculation was used for the test conditions:

$$\text{Cytotoxicity (\%)} = (\text{experimental value/cell death control value}) \times 100$$

## SUPPLEMENTAL FIGURE LEGEND

**Figure E1.** Fibroblasts in non-contact co-culture do not mimic the macrophage modulation effects of mesenchymal stromal cells (MSCs) in the presence of lipopolysaccharide (LPS). Fibroblasts are able to reduce TNF- $\alpha$  production by LPS-treated monocyte-derived macrophages (MDMs) (A) but have no effect on IL-8 production (B) (n=3 per group). Fibroblasts do not increase the expression of M2 macrophage marker CD206 (C) (n=4 per group). All one-way ANOVA with Bonferroni's post-hoc test. Data are presented as mean  $\pm$  standard deviation.

**Figure E2.** Mesenchymal stromal cells (MSCs) have no effect on total IL-8 levels in bronchoalveolar lavage fluid (BALF)-treated monocyte-derived macrophages (MDMs) (n=2 per group). Data are presented as mean  $\pm$  standard deviation.

**Figure E3.** Rhodamine-6G pre-treatment of human mesenchymal stromal cells (MSCs) inhibits mitochondrial function and has little effect on paracrine factor production. (A) After 48 hours of rhodamine pre-treatment, MSCs had dramatically reduced maximal mitochondrial respiratory capacity (i) and mitochondrial ATP turnover (ii), resulting in upregulation of non-mitochondrial respiration (iii) demonstrated by the Seahorse metabolic analyzer (n=1 (in triplicate) per group). Rhodamine pre-treatment resulted in a small reduction in the levels of angiopoietin-1 (B) and IL-8 (C) in MSC culture medium (n=2 (in triplicate) per group). Rhodamine treatment of MSCs had no cytotoxic effects at 48 hours (D) (n=1 per group). Data are presented as mean  $\pm$  standard deviation.

**Figure E4.** Oligomycin treatment for 24 hours does not have cytotoxic effects on monocyte-derived macrophages (MDMs) (n=2 per group). Data are presented as mean  $\pm$  standard deviation.

Fig E1

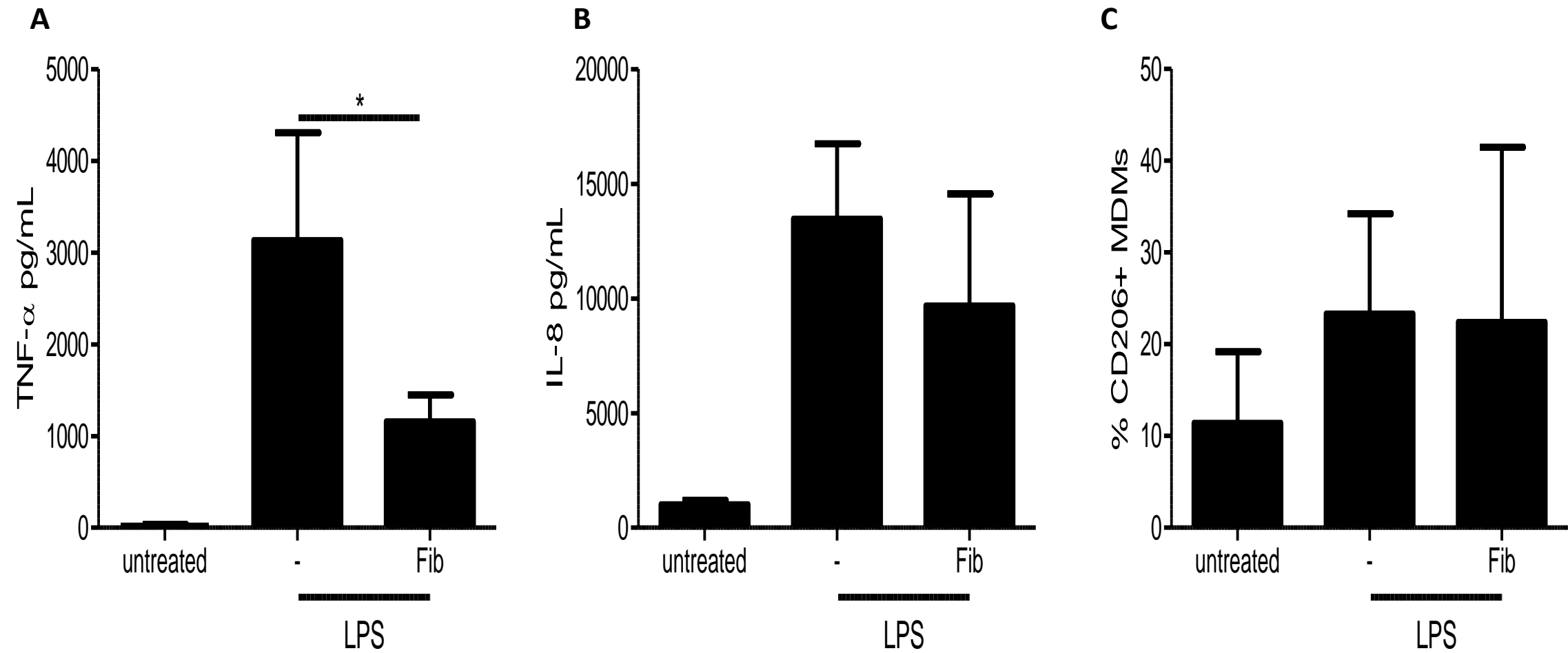




Fig E2

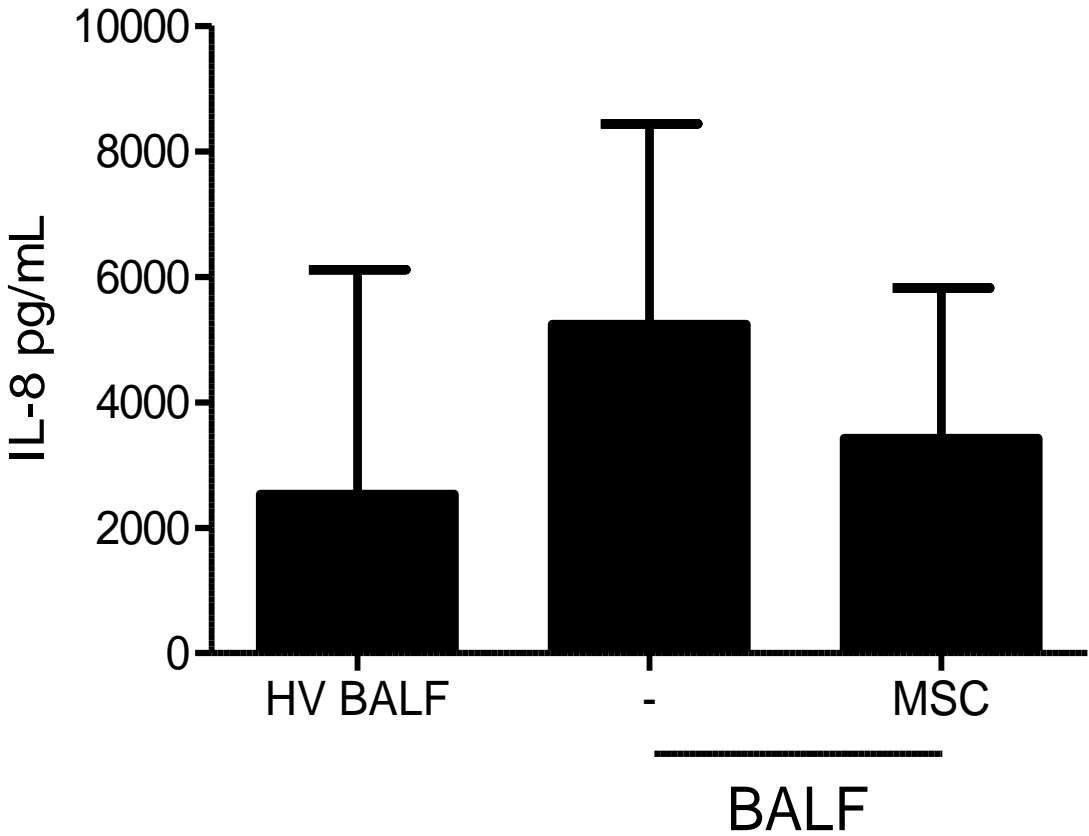


Fig E3

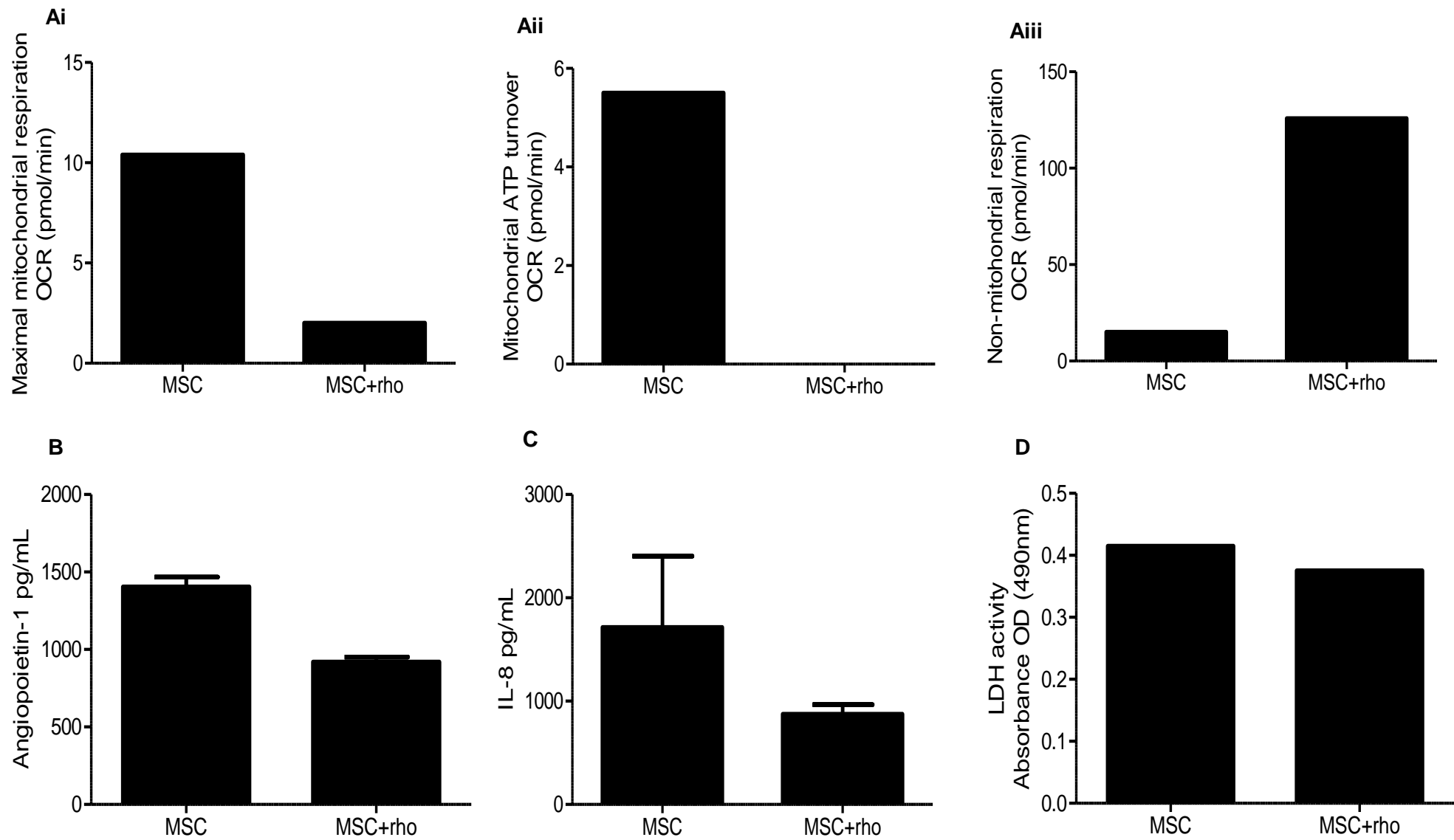


Fig E4

